CONTRASTING EFFECTS OF NITROGENOUS POLLUTION ON FITNESS AND SWIMMING PERFORMANCE OF IBERIAN WATERFROG, *PELOPHYLAX PEREZI* (SEOANE, 1885), LARVAE IN MESOCOSMS AND FIELD ENCLOSURES.

A. Egea-Serrano[a, 1], M. Tejedo[b]

[a] Departament of Zoology and Physical Anthropology. University of Murcia. 30100 Murcia (Spain). E-mail address: aegea@um.es

[b] Department of Evolutionary Ecology. Estación Biológica de Doñana, CSIC. Avda. Américo Vespucio s/n. 41092 Sevilla (Spain). E-mail address: tejedo@ebd.csic.es

Corresponding author: Andrés Egea-Serrano
Departament of Zoology and Physical Anthropology
University of Murcia, 30100 Murcia, SPAIN
Telephone number: +34 868 88 4961
Fax number: +34 868 88 3963
E-mail: aegea@um.es

[1] Present address: State University of Santa Cruz. Laboratory of Vertebrate Zoology. Pavilhão Max de Menezes, Campus Soane Nazaré de Andrade. Rodovia Jorge Amado, km 16, Bairro Salobrinho. CEP 45662-900 Ilhéus-Bahia (Brazil).

ABSTRACT

Amphibians are declining worldwide and pollutants have been implicated as a major contributor to these declines. To understand these declines, many studies have assessed the impact of pollutants on amphibian behavior, however information regarding their effect on locomotor abilities, as well as the intra-specific variation of the tolerance to pollutants, is extremely rare. Further, the majority of studies examining the impact of pollutants on amphibians have been conducted in simplified laboratory settings. Given the complexity of natural systems, determining whether amphibian responses in laboratory studies are generalizable in more realistic natural scenarios is critical. Towards this goal, this study assessed the impact of nitrogenous pollution on survival and fitness-related larval traits (growth, mass and swimming performance) for three populations of the frog *Pelophylax perezi*, exposed to different degrees of eutrophication in two different and complementary experiments: (1) pond mesocosms, with NH_4Cl isolated or combined with $NaNO_2$ and $NaNO_3$, and (2) field enclosures placed in natural streams differing in their degree of pollution.

For both mesocosm and field enclosure experiments, larval mortality was unaffected by nitrogenous pollution. However, in the mesocosm experiment, exposure to nitrogenous compounds reduced final larvae mass and growth. In contrast, in the enclosure experiment, polluted locations facilitated final mass and growth of surviving tadpoles. Population-level variation in the effect of pollution was observed for final larval mass in the mesocosm but not in the field enclosure experiment. In addition, although nitrogenous compounds in both mesocosm and natural conditions had no direct effect on absolute larval swimming performance, they may impact the viability of larvae by affecting the relationships between growth and the swimming abilities. The differential

pattern found in the impacts of nitrogenous compounds on larvae of *P. perezi* when raised in different experimental venues (mesocosms and field conditions) points to the convenience of considering more realistic natural scenarios in assessing the impact of pollutants on amphibians.

Key words: Nitrogenous pollution, experimental venue, inter-populational variation, amphibian larvae, fitness, swimming performance.

1. Introduction

Amphibian populations are declining worldwide (Gardner, 2001; Blaustein & Kiesecker, 2002). Although natural fluctuations may cause demographic variation (Pechmann *et al.*, 1991; Tejedo, 2003), chemical pollutants have been implemented as a major contributor to these declines (Scoccianti, 2001; Semlitsch, 2003). In particular, pollutants associated with agricultural practices have been linked to amphibian decline in multiple regions of the world (Berger, 1989; Davidson *et al.*, 2001, 2002; Hamer *et al.*, 2004). One of the effects of managing croplands is the increase of the environmental concentration of nitrogenous compounds (Ritter & Bergstrom, 2001). Such an increase is, at least partially, a consequence of the use of nitrogenous fertilizers. Because this type of fertilizers are widely used throughout the world (Carpenter *et al.*, 1998) and expected to be more present in the future (Tilman *et al.*, 2001; Galloway *et al.*¸ 2003), an increasing negative impact on the wildlife could be expected. Thus, understanding the impacts of increasing nitrogenous pollution on wildlife populations is paramount.

Within the past two decades, increasing studies have assessed the impact of nitrogenous compounds on amphibians (see review Marco & Ortiz-Santaliestra, 2009) and have demonstrated that compounds such as ammonium, nitrite and nitrate can significantly affect survival, mass, development, behaviour and even habitat occupancy of amphibians (e.g. Hecnar, 1995; Xu & Oldham, 1997; Marco *et al.*, 1999; Hatch & Blaustein, 2000; Smith *et al.*, 2005; Boone *et al.*, 2007; Griffis-Kyle, 2007; Macías *et al.*, 2007; Peltzer *et al.*, 2008; Shinn *et al.*, 2008, 2013; Egea-Serrano *et al.*¸ 2009, 2011, 2012; Smith & Dibble, 2012; Denoël *et al.*, 2013a). Additionally, amphibian responses to nitrogenous compounds can vary with high levels of inter- (Marco *et al.*, 1999; Shinn *et al.*, 2008, 2013) and intra-specific variation (Johansson *et al.*, 2001; Shinn *et al.*,

2008, 2013; Egea-Serrano *et al.*, 2009). Despite this growing body of work, to our knowledge, there are no studies assessing the effect of nitrogenous compounds on swimming performance of amphibians. Further, population-level variation in amphibian swimming performance in response to nitrogenous pollution has yet to be reported. Past studies have demonstrated negative effects of pollutants on swimming behaviours (pesticides, aluminium, road de-icing salts or acidity; Jung & Jagoe, 1995; Bridges, 1997; Widder & Bidwell, 2006, 2008; Denoël *et al.*, 2010, 2013b; Lavorato *et al.*, 2013), thus we hypothesize that nitrogenous compounds will also negatively affect swimming performance.

The majority of studies examining the impact of pollutants on amphibians have been conducted in simplified laboratory settings. While lab-based studies are a starting point in understanding the effects of a pollutant, natural systems are complex and amphibian responses to pollutants can be influenced by a number of factors and the potential for non-additive interactions among different pollutants (e.g., Boone *et al.*, 2005). Therefore, to provide ecological relevance to the conclusions derived from laboratory studies, it is useful to determine whether the effects recorded ensue in the field (Boone & Bridges, 2003; Boone & James, 2005). Pond mesocoms and field enclosures have been described as useful tools to study the effect of pollutants in realistic conditions (Boone & Bridges, 2003; Boone & James, 2005). Mesocosms have been defined as independent, closed outdoor artificial systems (either aquatic or terrestrial) containing food webs and processes representative of natural environment, whereas enclosures are defined as permeable containers enclosing the study organisms within a particular environment, allowing environmental exchange among enclosures (Boone & James, 2005). The characteristics of these methodological approaches allow to integrate multiple stressing factors in natural environments as well as to accurately

study population and community level processes (Boone & James, 2005). For instance, under mesocosm and enclosure conditions, algal growth may be affected by chemical pollution (e.g., Boone *et al.*, 2007; Relyea, 2009) and there is typically a reduction in larval crowding conditions in these experimental venues compared to laboratory studies (although in this last case designs considering individually kept experimental larvae may be performed, avoiding therefore any confounding crowding effect; e.g., Egea-Serrano *et al.*, 2009; Hangartner *et al.*, 2012). Because these facts may result into high algae availability and low competition level, pollutants may have indirect effects, via higher food availability per capita, on survival and/or growth that with difficulty can be taken into account in laboratory studies, because, as it is necessary to do to prevent experimental units from oxygen depletion and waste concentration, water is renewed frequently (which prevents the establishment of algal communities) and, commonly, experimental individuals larvae are not reared singly.

Exposure to multiple stressors (as expected for natural settings) may lead to unexpected additive or synergistic responses (Berenbaum, 1989). For example, the presence of low levels of salinity can reduce the toxicity of nitrite (Shinn *et al.*, 2013). In contrast, the presence of other chemicals (Boone *et al.*¸ 2005; Egea-Serrano *et al.*¸ 2009), UV-B radiation (Hatch & Blaustein, 2000, 2003; Macías *et al.*, 2007) or low pH (Hatch & Blaustein, 2000) can increase the severity of amphibian larval responses to nitrogenous pollutants. Since amphibians are commonly exposed to multiple pollutants in nature, assessing the consequences of nitrogenous compounds alone and in combination is essential to developing our understanding of amphibian responses to nitrogenous pollution. Yet, to date the majority of ecotoxicological studies have focused on understanding the effects of single pollutants (Storfer, 2003).

The mechanisms driving population-level trait expression can be complex, especially when selection acts upon correlated traits, such as growth and performance (Arnold, 1983; Arendt, 2003). A linkage between fitness-related traits has been described for both natural and polluted environments (e.g. Arendt, 2003; Egea-Serrano *et al.*, 2011; Denoël *et al.*, 2013b), which may suggests that the impact of pollution on a given trait may be the correlated response to other traits. Taking this fact into account, and considering that individual populations may show different tolerance to pollution (e.g., Johansson *et al.*, 2001; Shinn *et al.*, 2008, 2013; Egea-Serrano *et al.*, 2009), it is reasonable to hypothesize that the relationship between correlated traits will vary in polluted environments versus non-polluted environments. This hypothesis emphasizes the convenience of exploring the existing relationships between traits related to both fitness and performance to shed light on the mechanisms by which pollution, and particularly nitrogenous compounds, may affect amphibians.

The aims of this study were to: 1) determine the effects of high, but ecologically relevant, concentration of ammonium chloride, alone and in combination with sodium nitrite and sodium nitrate, on mortality, mass and growth, and swimming performance of larvae of *Pelophylax perezi* raised both in mesocosm conditions (*common garden experiment*) and in enclosures placed in natural streams differing in their level of nitrogenous pollution (*field experiment*); 2) evaluate whether differences exist among populations in their tolerance to nitrogenous pollution; and 3) analyse the impact of nitrogenous pollution on the trade-off between larval growth and swimming performance for both the common garden and field experiment.

2. Material and methods

2.1. Model organisms.

P. perezi is an anuran that is found throughout the Iberian Peninsula and Southern France (Llorente & Arano, 1997), mainly occupying permanent water bodies, such as ponds and streams (García-París *et al.*, 2004). These permanent habitats receive runoff from nearby croplands and livestock farms, which is one of the most important nitrogen sources in the nature (e.g. Ritter & Bergstrom, 2001), and can contain high concentrations of different nitrogen forms (e.g. for southeastern Iberian Peninsula: 154.6 mg NH_4^+/L; 74.4 mg NO_2^-/L; 333.0 mg NO_3^-/L, Suárez, personal communication). Both *P. perezi* juveniles and adults use these permanent water bodies as shelter from predators (Martín *et al.*, 2006), for foraging (Docampo & Vega, 1990), and breeding (Egea-Serrano *et al.*¸ 2005), thus this species is likely exposed to nitrogen pollution throughout its life cycle. In addition to being exposed to different types of nitrogenous inputs, *P. perezi* populations vary in their level of exposure to nitrogenous pollution and local adaption in tolerance to this pollution has been previously reported (Egea-Serrano *et al.*, 2009). Thus, this species is a good model for assessing the interpopulational variation and the effect of nitrogenous cocktails on amphibians.

2.2. Studied populations

We collected five different egg masses of *P. perezi* from three populations located in the Segura River Basin (SE Iberian Peninsula) in the first fortnight of March 2007. Water bodies within this basin face arid conditions (Vidal-Abarca *et al.*, 1987), and increasing eutrophication due to local farming practices (Ballester, 2003). For this study, we chose populations that were naturally exposed to different levels of nitrogen pollution. To

represent populations not exposed to nitrogenous pollution (reference populations), we chose *P. perezi* populations in Río Chícamo (38º12´N, 001º03´W; 170.3 m.a.s.l.), a permanent headwater stream, and Sierra Espuña Regional Park (37º52´N, 001º30´W; 673.0 m.a.s.l.), a seminatural pond (C1 and C2, hereafter). To represent a polluted population, we chose a *P. perezi* population in Rambla del Garruchal (37º57´N, 001º04´W; 346.0 m.a.s.l.), a semipermanent headwater stream (herafter P1). Detailed descriptions of C1 and P1 populations can be found in Egea-Serrano *et al.* (2009). Although no nutrient concentration data is available for C2, it is unlikely that amphibians were exposed to pollution because the area surrounding the location of this population is isolated from both urban and farming activities. The surrounding terrestrial environment of this population is composed of pine trees on limestone lithology. The geographical distance between populations ranged from 28.3 km to 54.9 km.

2.3. Experimental design and response variables

The developmental stage of collected embryos did not differ between populations (Chi-square, $P > 0.05$) and ranged from Gosner stage 15 to 18 (Gosner, 1960). In all cases, embryos were transported before hatching to the laboratory, where they were reared in 12 L glass aquaria containing dechlorinated tap water (pH = 8.39; conductivity = 985 µS/cm; 0.002 mg NO_2^-/L; 4.69 mg NO_3^-/L). We applied treatments once individuals reached Gosner´s stage 25.

2.3.1. Common garden experiment.

One month prior to the start of the experiment, we set up a total of 16 outdoor plastic pools (430 L of capacity) at the Campus Universitario de Espinardo (Universidad de Murcia, Spain). We filled each pool with 200 L of dechlorinated tap water, added a thin layer of leaf litter to each pool to serve as a natural resource for tadpoles, and placed a plastic tile (229 cm^2) facing the south side for later assessments of periphyton biomass (Relyea *et al.*, 2005). To initiate plankton communities, each pool was inoculated with 0.5 L of water from a local natural pond once a week before the experiment began. Prior to the start of the experiment (26 March 2007), we reduced the water volume in each pool by 150 L, to correct for differences in water volume due to evaporation. We randomly selected five larvae from each study site to add to each pool (i.e. a total of 15 larvae per pool). The number of larvae exposed to the treatments was selected from the available number of individuals at developmental stage 25 (Gosner, 1960), which was limited because of the observed asynchrony in the breeding season among the sampled populations and the low relative fecundity of the study species in the study area (Egea-Serrano, unpublished data). Since larvae used in this experiment came from different populations, each larva was individually placed in 1.0 L plastic beaker covered with 1 mm mesh lid within each pool. Initial mass of larvae did not differ in relation to their population of origin ($F_{2, 237} = 1.064$; $P = 0.347$).

We added one dog chow pellet (250-350 mg) to each beaker and larvae were allowed to acclimate to the pools for two days before the beginning of the experiment. No additional food was provided to larvae during the experiment. Each pool was randomly assigned to one of the four following treatments: 1) control; 2) 13.5 mg NH_4^+/L; 3) 13.5 mg NH_4^+/L + 364.7 mg NO_3^-/L + 6.67 mg NO_2^-/L; 4) 13.5 mg NH_4^+/L + 364.7 mg NO_3^-/L + 66.7 mg NO_2^-/L. These treatments were selected because they produced the highest larval mortality in the laboratory (Egea-Serrano *et al.*, 2009) and

because concentrations were well below within those naturally occurring in the field in the Segura River basin (e.g. 154.6 mg NH_4^+/L; 74.4 mg NO_2^-/L; 333.0 mg NO_3^-/L, Suárez, personal communication). To obtain the experimental concentrations, 40 g NH_4Cl/l, 70 g $NaNO_2$/l and 150 g $NaNO_3$/l stock solutions were poured directly into the pools. Such solutions were prepared using NH_4Cl, $NaNO_2$ and $NaNO_3$ salts that were 99%, 97-101%, and 99% pure, respectively. NH_4Cl salt was purchased from Prolabo (France). The rest of the salts were purchased from Panreac (Spain). Each treatment was replicated four times for a total of 16 experimental pools. The experiment thus consisted in a split-plot design (Quinn & Keough, 2002), where treatment was the main plot factor and the population of origin the subplot factor.

2.3.2. Field experiment.

Larvae from the three study populations (C1, C2, P1) were transplanted and reared in four different streams. To represent unpolluted locations, we chose: Rambla del Estrecho (37º46´N, 001º45´W; 476.7 m.a.s.l.), a permenant headwater stream, and Rambla Tejera (38º11´N, 002º07´W; 1197.9 m.a.s.l.), a semipermanent headwater stream. Both of these streams are located in forested environments with no or marginal traditional farming activities, therefore the presence of nitrogenous pollution in the water column is unlikely. During the exposure period nitrogenous compounds levels were (mean ± SE): Rambla del Estrecho: 0.6 mg NO_3^-/L ± 0.08, n = 3; Rambla Tejera: 1.4 mg NO_3^-/L ± 0.3, n = 4. To represent polluted environments, we chose locations in the permanent rivers: Río Quípar (38º02´N, 001º54´W; 710 m.a.s.l.) and Río Mula (38º03´N, 001º25´W; 190.2 m.a.s.l.). Both locations exposed to high degree of eutrophication from urban wastewaters and farming practices. During the exposure

period their nitrogenous compounds levels were (mean ± SE): Río Quípar: 25.02 mg NO_3^-/L ± 3.0, n = 4; Río Mula: 10.1 mg NO_3^-/L ± 1.1, n = 4. The presence of natural populations of *P. perezi* was confirmed in all of these streams.

We set up three enclosures at least 1 m apart at each of the four locations. Each enclosure was placed close to the stream shore, where shore vegetation grew, allowing for the existence of natural variation in light and UV-radiation throughout the day. While it is possible that the water quality, light, and planktonic communities varied between the enclosure and common garden experiments, other factors, such as environmental temperature or rainfall were roughly pretty similar across the whole study area (Egea-Serrano, unpublished data). To determine periphyton biomass, a 229 cm^2 plastic tile was placed into each enclosure facing the south side. We randomly selected and added two to three larvae collected from C1, C2, and P1 to each enclosure (i.e. a total of 6 – 9 larvae per enclosure). Similar to the common garden experiment, the number of larvae added to each enclosure was limited by the available number of individuals at developmental stage 25 (Gosner, 1960). Initial mass of larvae did not differed in relation to their population of origin ($F_{2,69}$= 1.936; P= 0.152). In order to assess population-level variation, larvae were individually placed in 1.5 L plastic beakers covered with 1 mm mesh lid. Larvae were fed one dog chow pellet (250-350 mg) at the beginning of the experiment and no food was provided during the rest of the experiment. Due to an increase in water turbidity after a heavy rain event, all larvae in the Rambla Tejera enclosures died. Thus, this location was removed from the subsequent statistical analyses. Therefore, the design for the field experiment consisted of one reference site (Rambla del Estrecho, L1 hereafter) and two polluted sites (Río Quípar and Río Mula, hereafter L2 and L3, respectively).

2.3.3. Response variables.

For both the common garden experiment and field experiment, after 21 d of exposure, we assessed larval mortality and mass of surviving larvae (±0.1 mg). We also calculated instantaneous larval growth (change in mass over 21 days of exposure: final mass-initial mass; Arendt, 2003). Finally, since larval swimming speed, an ecological measure of locomotor performance, provides information on larval potential fitness (Huey & Stevenson, 1979; Denoël *et al.*, 2013b), we measured individual swimming speed on day 22 after the beginning of the experiment. To measure swimming speed, we placed an individual larva into a plastic aquarium (length: 40 cm; width: 1 cm) and chose a water depth (~ 2 cm) that completely covered the larvae but limited swimming to a two-dimensional space. After a one-minute acclimation period, the tail of the larva tail of the larva was stroked with a glass rod to induce a swimming response. This technique has been commonly used to elicit startle response in amphibian larvae (e.g., Brown & Taylor, 1995; Bridges, 1997; Van Buskirk & McCollum, 2000; Arendt, 2003). Swimming was videotaped (Olympus FE-200 digital compact camera) overhead until swimming was completed (i.e. tail clearly stopped beating) (Bridges, 1997). Total swimming speed during a hypothetical escape event was estimated as total distance swam/time swimming (cm/s). For both the common garden and field experiment, measurements of swimming speed were performed in laboratory conditions immediately after larvae were removed from the experimental units. Laboratory conditions held constant with temperatures ranging from 24-25ºC, and natural photoperiod and lighting coming from close windows. Once they were removed, larvae were kept in water collected from the pools and field sites where they had been reared. Videotaping was carried out between 8 a.m. and 3 p.m.

2.3.4. Environmental measurements.

On days 2 (day 0 for the field experiment), 7, 14 and 21 of the experiment, we measured several water physicochemical characteristics (pH, temperature [ºC], conductivity [µS/cm], salinity [g/l] and depth [cm]) in each pool and at each field location. At the same time, a water sample was taken from each pool and field location to determine the ammonium, nitrite and nitrate concentrations in the water column. Ammonium and nitrite analyses were performed by colorimetric methods whereas nitrate concentration was estimated by ionic chromatography. At the end of the experiment, since periphyton availability can affect tadpole growth and performance (Boone *et al.*, 2005, 2007), we measured periphyton biomass as an estimate of the supplemental resource available to the tadpoles in each pool. We assessed periphyton biomass by scraping periphyton off the surface of each tile. Once the periphyton obtained was dried allowing the water to evaporate, it was weighed (± 0.1 mg). In the case of the field experiment, periphyton growth was so scarce that its biomass could not be differentiated from sediments that had adhered to the periphyton tiles. To avoid any bias due to the weighing of a mix of periphyton and sediments, periphyton biomass was not considered for the field experiment.

2.4. Data analysis

2.4.1. Common garden experiment.

Larval mortality was analysed using generalized nonlinear model (GLZ) for binary data fitting a binomial distribution of the data with a Logit Link function. Population of origin, treatment and population x treatment interaction were considered as fixed factors and pool (nested within treatment) was considered as a random factor. Additionally, considering the hypothesis suggesting that sensitivity may be affected by larval initial size (since it may be correlated with egg size and, thus, roughly reflecting a maternal induced effect; Kaplan & Phillips, 2006), initial mass was considered as a covariate. GLZ was implemented in R 2.15.0 using lmer and lmerTest (Baayen *et al.*, 2008).

We analyzed larval mass using an ANCOVA, where population of origin, treatment, and their interaction, were treated as fixed factor. In this analysis we treated pool (nested within treatment to correct for the spatial heterogeneity in the experiment) as a random factor, and initial mass as a covariate. We analyzed growth using an ANOVA, and treated each factor similarly to that described in the analysis of larval mass. Since growth is a composite variable involving both initial and final mass, we did not include any covariate regarding either initial or final size in the analysis.

We analyzed larval swimming speed using an ANCOVA and treated population of origin, treatment, and their interaction as fixed factors, pool (nested within treatment) as random factor, and final mass as a covariate.

For all significant ANOVAs results, we conducted pairwise comparison tests using HDS Tukey´s tests to differentiate the effects of population of origin or treatment. For significant ANCOVAs, we conducted pairwise comparisons using estimated marginal means since post hoc tests are disabled when covariates are present in the model.

The existence of a trade-off between growth (independent predictor) and swimming speed (dependent variable) (Arendt, 2003) was analysed by stepwise regression analyses. Prior to the regression analyses, homogeneity of slopes assumption

was tested by ANCOVA to compare the regression lines between groups. In such ANCOVA, swimming speed was considered as the dependent variable and population of origin and treatment were included as fixed factors, pool (nested within treatment) as a random factor and larval growth as a covariate. All the interactions between the covariate and the fixed factors were also included in the analysis. When the influence of growth on swimming speed differed across population and/or treatment, separate regression analyses were performed for each population and/or treatment.

All variables were log-transformed. Statistical analyses of mass, growth and swimming performance were carried out using the statistical software SPSS® v. 15.0. Additionally, post hoc power analyses for ANOVA, ANCOVA (when the model included a covariate) or linear regression (in the case of the analysis of the trade-off between growth and swimming speed) were performed on larval traits using G*Power 3.1 (Faul *et al.*, 2007, 2009) and selecting $\alpha = 0.05$.

2.4.2. Field experiment.

Data transformation, statistical analyses and models for such analyses were similar to those conducted for the common garden experiment. In the field experiment analyses, location (fixed factor) and enclosure (random factor nested within location) replaced the treatment (fixed factor) and pool (random factor nested within treatment) of the common garden experiment.

2.4.3. Environmental measurements

For the common garden experiment, water physicochemical variables, nitrogenous ion concentrations and final periphyton biomass were analysed using separate ANOVAs. Treatment (for all variables), time of measurement (only for physicochemical variables) and their interaction (only for physicochemical variables) were considered as fixed factor and pool as random factor nested within treatment (for all variables). However, due to a shortage of samples with detectable values for the nitrogenous ion concentrations, for each pool, data belonging to different time points were pooled. Consequently, time of measurement effect could not be estimated and was not longer considered in our design. Because periphyton biomass was estimated only at the end of the common garden experiment, time of measurement was also excluded from the statistical analysis of the effects of treatment on periphyton biomass. Analyses were conducted on log-transformed data using the statistical software SPSS® v. 15.0. The analysis of physicochemical variables and nutrient concentration for the field experiment was performed identically as in the case of the field experiment, except for location (fixed factor) replaced the fixed factor treatment where appropriate. Since in this case estimates for these variables were taken outside enclosures, the factor enclosure (nested within location) was not considered in the statistical analyses.

3. RESULTS

3.1. Common garden experiment

We found low overall larval mortality (mean [±1 SE] = 7.088 ± 1.769%; n = 16; range: 0.0 – 20%) and mortality following exposure to nitrogenous compounds alone and in combination did not differed across treatments or populations ($P \geq 0.384$ in all

cases, results not shown). Mixtures of nitrogenous compounds reduced final mass and growth for all studied populations (Table 1; Fig. 1). We found a significant effect of population of origin on final mass (Table 1), and pairwise analyses indicated that larvae from C2 were larger than those from the other populations. We also found a significant interaction between population of origin and treatment for final mass (Table 1). Larvae from population C1 and C2 had reduced mass when exposed to treatment 2 (40 mg NH_4Cl/L), whereas the mass of larvae from the polluted population (P1) did not change.

Our analysis of swimming speed indicated no significant effect of treatment, population of origin, or their interaction ($P \geq 0.280$ in all cases, results not shown). Supplementary Table 4 shows the results of the ANCOVAs performed to test the homogeneity of slopes for the growth-swimming analyses. The relationship between larval speed and growth differed across treatments. Growth was negatively correlated with swimming speed in the control treatment, positively correlated in the ammonium enriched treatment (treatment 2), and marginally positively correlated in combined nitrogenous compounds (treatments 3 and 4) (Table 2; Fig. 2). In addition, swimming speed did not differ among populations of origin and treatments. Thus, the results found in the regression analyses are therefore likely driven by the different growth rates across populations and treatments.

Statistical power for the response variables was overall medium-large, except for the interaction between population of origin and treatment in the case of larval mortality, growth and swimming speed (Supplementary Table 5). Nevertheless, in these cases, the interaction effect exhibited high *p* – value that does not support the hypothesis of the existence of a significant effect.

3.2. Field experiment

We found moderately low mortality (mean [±1 SE]: 17.00 ± 5.11%; n = 9; range: 0.0 - 44.4%) but mortality was higher at the polluted location L3 (Fig. 3). However, none of the factors included in the analysis had a significant effect ($P \geq 0.187$ in all cases, results not shown). Final mass and growth was higher for the polluted L3 location than for L1 and L2 locations (Table 1; Fig. 4).

We found no significant main effect of population of origin or location on swimming speed. Additionally, the effect of the different experimental locations on swimming performance was not affected by the population of origin ($P \geq 0.293$ in all cases, results not shown).

We found a significant effect of growth on swimming speed across locations (Supplementary Table 9). Similar to the trend found in the mesocosms, growth was positively correlated with swimming speed in the polluted location L2. In contrast, in the control location (L1), we found a marginally significant negative correlation between growth and swimming speed (Table 2; Fig. 5). Since swimming speed did not differ among populations of origin and locations, these contrasting covariation patterns are therefore likely driven by the different growth rates across populations and locations.

Power to detect a significant effect when it exists was overall medium-large for the response variables, except for, particularly, the main effect of population of origin on swimming speed and for the interaction between population of origin and location in the case of larval mortality, growth and swimming speed (Supplementary Table 5). Nevertheless, in these cases, *p* – values were high, therefore not supporting the hypothesis of the existence of a significant effect.

3.3. Environmental measurements

For the common garden experiment, water physicochemical variables significantly differed across time. With the exception of water depth and temperature, we also found a significant time by treatment interaction (Supplementary Tables 1, 2). Treatments corresponding to the combination of different nitrogenous compounds showed higher values for pH than control treatment, as well as higher conductivity and salinity than the rest of treatments (Supplementary Tables 1, 2). Furthermore, these differences were increased with time for pH and reduced for conductivity and salinity (Supplementary Tables 1, 2).

Polluted treatments significantly increased ammonium, nitrite and nitrate concentrations present in the water column in relation to control treatment (Supplementary Tables 2, 3). Moreover, in the case of nitrite and nitrate, the combination of nitrogenous compounds increased their concentration in relation to the rest of treatments (Supplementary Table 2).

We found a significant effect of the treatments on periphyton mass in the pools ($F_{3,12}$=23.341; P= 0.0001) with greater periphyton mass in pools exposed to nitrogenous compounds (Supplementary Table 2).

For the field experiment, physicochemical characteristics of water were significantly affected by time and location (Supplementary Table 6). L3 showed the highest values for all measured variables, except for water depth and pH (Supplementary Table 7). We also found a significant time by location interaction for all the physicochemical variables analysed (Supplementary Table 6). With the exception of water temperature and depth, differences in the physicochemical variables among

locations decreased during the experiment but increased again by the end of the experiment (Supplementary Table 7).

Due to the low levels of ammonium detected, we were unable to analyze these ion concentrations across locations. For nitrite concentration, only L2 and L3 had reliable data and L3 showed higher nitrite concentration. Nitrate concentration in the L2 location was the highest. L1 showed the lowest value for nitrate concentration (Supplementary Tables 7, 8).

4. DISCUSSION

Our main conclusion is that exposure to nitrogen-polluted environments, both in experimental mesocosms and natural field locations, resulted in stressful conditions that affected the final size, growth and the trade-off between growth and swimming speed of *P. perezi* larvae. The direction of the response initiated by the nitrogenous pollution treatments differed between the common garden and the field experiment. This differential pattern points to the benefit of integrating both venues in order to provide a more precise and accurate evaluation of the impact of nitrogenous pollution on *P. perezi* tadpoles.

In a meta-analysis, Egea-Serrano *et al.* (2012) reported that the overall impact of chemicals on amphibian survival was more severe when experiments were performed in enclosure conditions compared to mesocosms. This may be attributable to the higher synergies between pollution and other factors presumably operating in field locations in relation to other experimental venues (Egea-Serrano *et al.*, 2012). Interestingly, since neither enclosure nor mesoscosm experiments resulted in a significant effect on larval mortality, the results from our study do not support this general pattern. Although the

fluctuation of water physcochemical variables was overall low (as inferred from the standard errors reported in the Supplementary Table 6), mean water salinity, pH and temperature was higher for the polluted locations. Water salinity has been reported to increase larval mortality either singly or in combination with nitrogenous compounds (Karraker, 2007; Karraker & Ruthig, 2009; Karraker *et al.*, 2008, 2010; Ortiz-Santaliestra *et al.*, 2010), thus we expected high mortality rates in the polluted locations. Moreover, the main effect of pH or temperature, or their interaction with nitrogenous compounds, cannot be discarded, either. However, we found no effect of field location on larval mortality, suggesting that both nitrogenous ion concentrations and level of salinity (or of any other factor) was too low relative to the sensitivity of *P. perezi* larvae. It is also possible that such moderate level of salinity may confer a certain tolerance to nitrogenous compounds (Shinn *et al.*, 2013). Since the nominal concentrations used in the common garden experiment significantly increased larval mortality in laboratory conditions (Egea-Serrano *et al.*, 2009), the observed tolerance of *P. perezi* in the common garden experiment was not necessarily the consequence of a highly effective detoxification pathway in the absence of other stressing factors. An alternative explanation for the lack of effect on larval mortality is that periphyton growth was enhanced due to enriched ammonium, nitrite and nitrate concentrations (Camargo & Alonso, 2006) potentially reducing the concentrations of nitrogenous ions that larvae were exposed to. However, caution is advised when interpreting the results reported, since the sample size for both the common garden and, specifically, the field experiment was low.

Past studies found that the overall effect of chemicals on mass did not differ between mesocosm and enclosure venues (Egea-Serrano *et al.*, 2012). However, the pattern found in the present experiments for nitrogenous compounds differed from such

an overall trend. Previous experiments performed in mesocosm conditions have revealed either the absence of significant effects (Boone *et al.*¸ 2005) or the existence of positive influence of nitrogenous treatments on amphibian larvae growth (de Wijer *et al.*, 2003; Hatch & Blaustein, 2003; Boone *et al.*¸ 2007; Smith & Dibble, 2012). In contrast, in our common garden experiment, the combinations of nitrogenous compounds strongly reduced both mass and growth of *P. perezi* larvae. Considering that periphyton biomass was greater at the polluted treatments, we expected larval growth to be facilitated relative to control, as it has been previously suggested in other mesocosm settings (Boone *et al.*, 2005; 2007). Egea-Serrano *et al*. (2009) reported, for laboratory experiments, a lower feeding efficiency and subsequent decline in growth rate for *P. perezi* larvae at polluted treatments. Thus, the pattern we observed in the mesocosm study may be due to decreased feeding efficiency. While, nitrogenous pollutants decreased larval mass and growth in laboratory and mesocosm conditions, we detected the reversed pattern in the field experiment. Larvae exposed to natural nitrogenous pollution levels had higher final mass and growth at one of the polluted locations (L3). This result can be due to an indirect effect of pollution via food web (de Wijer *et al.*¸ 2003). Although periphyton biomass could not be properly measured at the field locations, it is likely that polluted sites would show high algal growth, since nutrient concentration was significantly higher than control site (see Camargo & Alonso, 2006). Since actual nitrogen concentration was lower in the field locations than in the experimental mesocosms and, likely, beakers used in the laboratory by Egea-Serrano *et al.* (2009), larvae were likely lightly affected by pollution and could be able to perform an overcompensation response to the stress produced by nitrogenous compounds by increasing, for instance, their feeding efficiency (Egea-Serrano *et al.*, 2011), which allowed to utilize the greater periphyton resource and achieve a higher mass. However,

we have to be cautious with this conclusion since in the present study only one reference field location was considered. These results may be biased if this location had unnoticed unique characteristics (e.g., higher predator density, higher occurrence of parasites) that also influenced amphibian larvae. Therefore, further studies involving a higher number of replicates for both reference and polluted field locations are required to identify whether the results obtained in this study are the consequence of nitrogenous pollution or whether the results were site specific.

Exposure to nitrogenous compounds did not affect swimming speed for either the mesocosm or field experiments. Since burst speed is assumed to be related with greater survival in larval anurans (Jung & Jagoe, 1995) and some direct evidences corroborate this prediction (Watkins, 1996; Kaplan & Phillips, 2006; Denoël *et al.*, 2013b), our results suggest that individual susceptibility to predators may not be affected by the effects of nitrogenous pollution, since larval escape ability is not modified either by treatments or by environments differing in their level of pollution. However, tadpoles reared in polluted environments could be indirectly exposed to a higher risk of predation because some larval amphibians increase their activity through the water column when exposed to nitrogenous pollution (Egea-Serrano *et al.*, 2011), which would increase predator encounter rates.

The lack of effect of nitrogenous compounds on swimming speed differs from past publications which report that chemicals can have significant effects on the presence of swimming patterns, lead to high irregular swimming rates, lower distance moved, and alter time showing swimming activity and swimming speed (Jung & Jagoe, 1995; Berril *et al.*, 1998; Bridges, 1997; Brunelli *et al.*, 2009; Denoël *et al.*, 2010, 2012, 2013b, Westman *et al.*, 2010; Egea-Serrano *et al.*, 2011; but see Widder & Bidwell, 2006, 2008). Chemicals such as salts, heavy metals or pesticides can cause osmotic

stress or interfere in the normal activity of given enzymes (e.g. cholinesterase), leading to harmful consequences on the nervous system and, therefore, affecting the transmission of the nervous impulse (Trainer & Karstad, 1960; Barron & Woodburn, 1995; Barabasz *et al.*, 2002). In contrast, nitrogenous compounds are not known to produce these responses in amphibians, which suggest that these compounds are less prone to affect swimming performance than other pollutants. Nevertheless, while there is limited data on amphibians, in fish, nitrite can affect the enzyme activities responsible for the transmission of nerve impulses indirectly limiting locomotor performance (Das *et al.*, 2004). Thus, we predicted a similar decline in tadpole locomotor performance in polluted areas. However, our results (once corrected for larval mass) did not support this hypothesis. These results are likely due to low concentrations of nitrogenous pollutants as their effects on enzyme activity were not strong enough to affect larval motor skills (see discussion in Widder & Bidwell [2006, 2008] for the effect of the pesticide chlorpyrifos). Further physiological research is needed to understand the pathways underlying this lack of effect of nitrogenous pollution on swimming performance of larvae of *P. perezi*.

Significant trade-offs between growth and swimming speed have been detected for some of the treatments or locations considered in the common garden and the field experiment, respectively. Arendt (2003) predicted that across taxa, there would be a generalizable negative relationship between growth and swimming speed. Billerbeck *et al.* (2001) demonstrated a tradeoff between higher growth rates and available energy for locomotion. The combination of this trade-off and the high energetic demands of detoxification (Wright & Wright, 1996), suggests that we should observe a negative relationship between growth and speed when larvae were exposed to pollution both in mesocoms and in the field. However, the expression of the negative correlation between

growth and swimming performance was only confirmed in the unpolluted control treatment and location (L1). In contrast, exposure to both high concentration of ammonium acting isolated (treatment 2, 13.5 mg NH_4^+/L) and to polluted permanent rivers (i.e. L2) produced a significant positive relationship between swimming speed and growth. Our results may be explained by van Noordwijk & de Jong´s model (van Noordwijk & de Jong, 1986), which suggests that if the variation in the total amount of energy acquired is large and the variation in energy allocation is small, positive correlations between traits could be expected. The lack of a trade-off at the polluted environments may be due to a high individual-level variation in the amount of resources acquired and invested in growth or in propelling structures and/or muscle development. This fact suggests that the capability to escape from predators depends on how to get larval size, and that the costs of rapid growth are environmentally dependent. Nevertheless, since both growth and swimming speed are composite variables, it is possible that factors that have not been taken into account may underlie the observed trade-offs (Arendt, 2003).

Although for most of the interactions between factors the statistical power was low, we found a significant population by treatment interaction for larval final mass in the common garden experiment. Thus, similar to past studies, these results suggest the existence of population-level variation and potentially local adaptation to pollutants (Hecnar, 1995; Johansson *et al.*, 2001; Hatch & Blaustein, 2003; Shinn *et al.*, 2008, 2013; Egea-Serrano *et al.*, 2009, 2011). However, the results obtained for the field experiment did not fit within the local adaptation hypothesis since populations did not differ in their response when transplanted into different locations. This could be attributed to the fact that natural environments were not stressful enough to magnify the different responses of larvae from each population to pollution. Nevertheless, just one

of the populations of origin corresponded to a polluted environment, being possible that larvae from this population performed differently due to causes not related to the adaptation to pollution. Consequently, further studies where populations of origin corresponding to both reference and polluted environments are replicated in a higher number are needed to determine whether the population-specific response detected, at least for larval mass in the common garden experiment, is driven by nitrogenous pollution or by any other factor.

In conclusion, larval *P. perezi* is sensitive to nitrogenous pollution in semi-natural and natural environments since both fitness and the trade-off between growth and swimming speed were clearly affected. Mesocosm and enclosure venues led to different amphibian responses to nitrogenous pollutants but an important issue to consider is that the actual nitrogen concentrations found in each venue and the interaction between pollution and other factors differed. This fact should be considered in future research to contribute to provide ecologically relevant conclusions on the impact of nitrogenous compounds and, more generally, chemical pollution on amphibians.

Acknowledgements

We thank Domingo Campillo for authorizing the use of the facilities located at the drinking water treatment station of Contraparada (Empresa Municipal de Aguas y Saneamiento de Murcia, S.A.) to determine the concentrations of ammonium, nitrite and nitrate in both the experimental pools and field locations. We are indebted to Isabel Hurtado for her invaluable assistance during the performance of such determinations. We are thankful to two anonymous reviewers for their comments on an early draft of the manuscript. We are also indebted to Jessica Hua for kindly checking English

grammar and style. A.E.S. was funded by a Caja de Ahorros delMediterráneo predoctoral fellowship and is currently supported by a postdoctoral fellowship granted by the Conselho Nacional de Desenvolvimento Científico y Tecnológico – CNPq of Brazil (ref. 370592/2013-1).

Appendix A.

Supplementary data associated with this article.

References

Arendt, J.D. 2003. Reduced burst speed is a cost of rapid growth in anuran tadpoles: problems of autocorrelation and inferences about growth rates. Functional Ecology, 17: 328-334.

Arnold, S.J. 1983. Morphology, performance and fitness. American Zoologist, 23: 347-361.

Baayen, R. H., D. J. Davidson, and D. M. Bates. 2008. Mixed effects modeling with crossed random effects for subjects and items. Journal of Memory and Language, 59: 590-412.

Ballester, R. (dir). 2003. *Los humedales de la Región de Murcia: Humedales y ramblas de la Región de Murcia*. Dirección General del Medio Natural. Consejería de Agricultura, Agua y Medio Ambiente, Región de Murcia.

Barabasz, W.; Albińska, D.; Jaśkowska, M. & Lipiec, J. 2002. Ecotoxicology of aluminium. Polish Journal of Environmental Studies, 11: 199-203.

Barron, M.G. & Woodburn, K.B. 1995. Ecotoxicology of chlorpyrifos. Reviews of Environmental Contamination and Toxicology, 144: 1–93.

Berenbaum, M.C. 1989. What is synergy? Pharmacological Reviews, 41: 93-141.

Berger, L. 1989. Dissapearance of amphibian larvae in the agricultural landscape. Ecology International Bulletin, 17: 65-73.

Berrill, M.; Coulson, D.; McGillivray, L. & Pauli, B. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. Environmental Toxicology and Chemistry, 17: 1738-1744.

Billerbeck, J.M.; Lankford, T.E. Jr. & Conover, D.O. 2001. Evolution of intrinsic growth and energy acquisition rates. I. Trade-offs with swimming performance in *Menidia menidia*. Evolution, 55: 1863-1872.

Blaustein, A.R. & Kiesecker, J.M. 2002. Complexity in conservation: lessons from the global decline of amphibian populations. Ecology Letters, 5: 597-608.

Boone, M.D. & Bridges, C.M. 2003. Effects of pesticides on amphibian populations, in: Semlitsch, R.D. (ed.), *Amphibian Conservation*. Smithsonian, Washington, pp. 152-167

Boone, M.D. & James, S.M. 2005. Aquatic and terrestrial mesocosms in amphibian ecotoxicology. Applied Herpetology, 2: 231-257.

Boone, M.D.; Bridges, C.M.; Fairchild, J.F. & Little, E.E. 2005. Multiple sublethal chemicals negatively affect tadpoles of the green frog, *Rana clamitans.* Environmental Toxicology and Chemistry, 24: 1267-1272.

Boone, M.D.; Semlitsch, R.D.; Little, E.E. & Doyle, M.C. 2007. Multiple stressors in amphibian communities: effects of chemical contamination, bullfrogs, and fish. Ecological Applications, 17: 291-301.

Bridges, C.M. 1997. Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. Environmental Toxicology and Chemistry, 19: 1935-1939.

Brown, R.M. & Taylor, D.H. 1995. Compensatory escape mode trade-offs between swimming performance and maneuvering behavior through larval ontogeny of the wood frog, *Rana sylvatica*. Copeia, 1995: 1–7.

Brunelli, E.; Bernabò, I.; Berg, C.; Lundstedt-Enkel, K.; Bonacci, A. & Tripepi, S. 2009. Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behaviour in *Bufo bufo* tadpoles. Aquatic Toxicology, 91: 135-142.

Camargo, J. A. & Alonso, A. 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environmental International, 32: 831–849.

Carpenter, S.; Caraco, N.F.; Correll, D.L.; Howarth, R.W.; Sharpley, A.N. & Smith, V.H. 1998. Nonpoint pollution of surface waters with phosphorous and nitrogen. Ecological Applications, 8: 559-568.

Das, P.C.; Ayyappan, S.; Das, B.K. & Jena, J.K. 2004. Nitrite toxicity in Indian major carps: sublethal effect on selected enzymes in fingerlings of *Catla catla, Labeo rohita* and *Cirrhinus mrigala*. Comparative Biochemistry and Physiology, Part C, 138: 3-10.

Davidson, C.; Shaffer, H.B. & Jennings, M.R. 2001. Declines of the California red-legged frog: climate, UV-B, habitat and pesticides hypothesis. Ecological Applications, 11: 464-479.

Davidson, C.; Shaffer, H.B. & Jennings, M.R. 2002. Spatial tests of the pesticide drift, habitat destruction, UVB, and climate-change hypotheses for California amphibian declines. Conservation Biology, 16: 1588-1601.

Denoël, M.; Bichot, M.; Ficetola, G.F.; Delcourt, J.; Ylieff, M.Y.; Kestemont, P. & Poncin, P. 2010. Cumulative effects of a road de-icing salt on amphibian behavior. Aquatic Toxicology, 99: 275-280.

Denoël, M.; D´Hooghe, B.; Ficetola, G.F.; Brasseur, C.; De Pauw, E.; Thomé, J.P. & Kestemont, P. 2012. Using sets of behavioral biomarkers to assess short-term effects of pesticide: a study case with endosulfan on frog tadpoles. Ecotoxicology, 21: 1240-1250.

Denoël, M.; Perez, A.; Cornet, Y. & Ficetola, G.F. 2013a. Similar local and landscape processes affect both a common and a rare newt species. PLoS ONE 8: e62727. doi:10.1371/journal.pone.0062727

Denoël, M.; Libon, S.; Kestemont, P.; Brasseurc, C.; Focant, J.F.; De Pauw, E. 2013b. Effects of a sublethal pesticide exposure on locomotor behavior: A video-tracking analysis in larval amphibians. Chemosphere, 90: 945-951.

de Wijer, P.; Watt, P.J. & Oldham, R.S. 2003. Amphibian decline and aquatic pollution: Effects of nitrogenous fertiliser on survival and development of larvae of the frog *Rana temporaria*. Applied Herpetology, 1: 3-12.

Docampo, L. & Vega, M.M. 1990. Contribución al estudio de la alimentación de *Barbus bocagei* (Steindachner, 1866), *Phoxinus phoxinus* (Linnaeus, 1758) y *Rana perezi* (Seoane, 1885) en ríos de Bizkaia. Scientia Gerundensis, 16: 61-73.

Egea-Serrano, A.; Oliva-Paterna, F.J. & Torralva, M. 2005. Selección de habitat reproductor por *Rana perezi* Seoane, 1885 en el NO de la Región de Murcia (SE Península Ibérica). Revista Española de Herpetología, 19: 113-125.

Egea-Serrano, A.; Tejedo, M. & Torralva, M. 2009. Populational divergence in the impact of three nitrogenous compounds and their combination on larvae of the frog *Pelophylax perezi* (Seoane, 1885). Chemosphere, 76: 869-877.

Egea-Serrano, A.; Tejedo, M. & Torralva, M. 2011. Behavioral responses of the Iberian waterfrog, *Pelophylax perezi* (Seoane, 1885), to three nitrogenous compounds in laboratory conditions. Ecotoxicology, 20: 1246-4257.

Egea-Serrano, A.; Relyea, R.; Tejedo, M. & Torralva, M. 2012. Understanding of impact of chemicals on amphibians: a meta-analytic review. Ecology and Evolution, 2: 1382-1397.

Faul, F.; Erdfelder, E.; Lang, A.-G. & Buchner, A. 2007. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behavior Research Methods, 39: 175-191.

Faul, F.; Erdfelder, E.; Buchner, A. & Lang, A.-G. 2009. Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. Behavior Research Methods, 41: 1149-1160.

Galloway, J.N.; Aber, J.D.; Erisman, J.W.; Seitzinger, S.P.; Howarth, R.W.; Cowling, E.B. & Cosby, B.J. 2003. The nitrogen cascade. Bioscience, 53: 341-356.

Gardner, T. 2001. Declining amphibian populations: a global phenomenon in conservation biology. Animal Biodiversity and Conservation, 24: 25-44.

García-París, M.; Montori, A. & Herrero, P. 2004. *Amphibia, Lissamphibia*. In: *Fauna Ibérica*, vol. 24. Ramos M.A. et al. (Eds.). Museo Nacional de Ciencias Naturales. CSIC. Madrid.

Gosner, K.L. 1960. A simplified table for staging embryos and larvae with notes on identification. Herpetologica, 16: 183-190.

Griffis-Kyle, K.L. 2007. Sublethal effects of nitrite on eastern tiger salamander (*Ambystoma tigrinum tigrinum*) and wood frog (*Rana sylvatica*) embryos and larvae: implications for field populations. Aquatic Toxicology, 41: 119-127.

Hamer, A.J.; Makings, J.A.; Lane, S.J. & Mahony, M.J. 2004. Amphibian decline and fertilizers used on agricultural land in south-eastern Australia. Agriculture, Ecosystems and Environment, 102: 299-305.

Hangartner, S.; Laurila, A. & Räsänen. 2012. The quantitative genetic basis of adaptive divergence in the moor frog (*Rana arvalis*) and its implications for gene flow. Journal of Evolutionary Biology, 25: 1587-1599.

Hatch, A.C. & Blaustein, A.R. 2000. Combined effects of UV-B, nitrate and low pH reduce the survival and activity level of larval Cascades frogs (*Rana cascadae*). Archives of Environmental Contamination and Toxicology, 39: 494-499.

Hatch, A.C. & Blaustein, A.R. 2003. Combined effects of UV-B radiation and nitrate fertilizer on larval amphibians. Ecological Applications, 13: 1083-1093.

Hecnar, S.J. 1995. Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from Southern Ontario. Environmental Toxicology and Chemistry, 14: 2131-2137.

Huey, R.B. & Stevenson, R.D. 1979. Integrating thermal physiology and ecology of ectotherms: a discussion of approaches. American Zoologist, 19: 357–366.

Johansson, M.; Räsänen, R. & Merilä, J. 2001. Comparison of nitrate tolerance between different populations of the common frog, *Rana temporaria.* Aquatic Toxicology, 54: 1-14.

Jung, R.E. & Jagoe, C.H. 1995. Effects of low pH and aluminum on body size, swimming performance, and susceptibility to predation of green tree frog (*Hyla cinerea*) tadpoles. Canadian Journal of Zoology, 73: 2171-2183.

Kaplan, R.H. & Phillips, P.C. 2006. Ecological and developmental context of natural selection: maternal effects and thermally induced plasticity in the frog *Bombina orientalis.* Evolution, 60: 142-156.

Karraker, N.E. 2007. Are embryonic and larval green frogs (*Rana clamitans*) insensitive to road deicing salt? Herpetological Conservation and Biology, 2: 35-41.

Karraker, N.E. & Ruthig, G.R. 2009. Effect of road deicing salt on the susceptibility of amphibian embryos to infection by water molds. Environmental Research, 109: 40-45.

Karraker, N.E.; Gibbs, J.P. & Vonesh, J.R. 2008. Impacts of road deicing salt on the demography of vernal pool-breeding amphibians. Ecological Applications, 18: 724-734.

Karraker, N.E.; Arrigoni, J. & Dudgeon, D. 2010. Effects of increased salinity and an introduced predator on lowland amphibians in Southern China: Species identity matters. Biological Conservation, 143: 1079-1086.

Lavorato, M.; Bernabò, I.; Crescente, A.; Denoël, M.; Tripepi, S. & Brunelli, E. 2013. Endosulfan effects on Rana dalmatina tadpoles: Quantitative developmental and behavioural analysis. Archives of Environmental Contamination and Toxicology, 64: 253-262.

Llorente, G.A. & Arano, B. 1997. *Rana perezi*, in: Pleguezuelos, J.M. (ed.), *Distribución y Biogeografía de los anfibios y reptiles en España y Portugal*. Asociación Herpetológica Española-Universidad de Granada, Granada, pp. 164-166.

Macías, G.; Marco, A. & Blaustein, A.R. 2007. Combined exposure to ambient UVB radiation and nitrite negatively affects survival of amphibian early life stages. Science of Total Envrionment, 385: 55-65.

Marco, A. & Ortiz-Santaliestra, M.E. 2009. Impact of reactive nitrogen on amphibians, in: Heatwole, H. & Wilkinson, J.W. (eds.), *Amphibian Biology, Vol. 8, Amphibian Decline: Diseases, Parasites, Maladies and Pollution*. Surrey Beatty & Sons, Baulkham Hills, New South Wales, Australia, pp. 3145-3185.

Marco, A.; Quilchano, C. & Blaustein, A.R. 1999. Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. Environmental Toxicology and Chemistry, 18: 2836-2839.

Martín, J.; Luque-Larena, J.J. & López, P. 2006. Collective detection in escape responses of temporary groups of Iberian green frogs. Behavioral Ecology¸ 17: 222-226.

Ortiz-Santaliestra, M.E.; Fernández-Beneítez, M.J.; Lizana, M. & Marco, A. 2010. Adaptation to osmotic stress provides protection against ammonium nitrate in *Pelophylax perezi* embryos. Environmental Pollution, 158: 934-940.

Pechmann, J.H.K.; Scott, D.E.; Semlitsch, R.D.; Caldwell, J.P.; Vitt, L.J. & Gibbons, J.W. 1991. Declining amphibian populations: the problem of separating human impacts from natural fluctuations. Science, 253: 892-895.

Peltzer, P.M.; Lajmanovich, R.C.; Sánchez-Hernández, J.C.; Cabagna, M.C.; Attademo, A.M. & Bassó, A. 2008. Effects of agricultural pond eutrophication on survival and health status of *Scinax nasicus* tadpoles. Ecotoxicology and Environmental Safety, 70: 185-197.

Quinn, G.P. & Keough, M.J. 2002. *Experimental design and data analysis for biologists*. Cambridge University Press, Cambridge.

Relyea, R. A. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159: 363–376.

Relyea, R.A.; Schoeppner, N.M. & Hoverman, J.T. 2005. Pesticides and amphibians: the importance of community context. Ecological Applications, 15: 1125-1134.

Richter-Boix A.; Llorente G.A. & Montori, A. 2006. Breeding phenology of an amphibian community in a Mediterranean area. Amphibia-Reptilia, 27: 544–549.

Ritter, W.F. & Bergstrom, L. 2001. Nitrogen and water quality, in: Ritter, W.F. & Shirmohammadi, A. (eds.), *Agricultural nonpoint source pollution: Watershed management and hydrology*. Lewis Publishers, Boca Ratón, pp. 59-90.

Scoccianti, C. 2001. *Amphibia: aspetti di ecologia della conservazione [Amphibia: Aspects of Conservation Ecology]*. Editore Guido Persichino Grafica. Firenze.

Semlitsch, R.D. 2003. Introduction: general threats to amphibians, in: Semlitsch, R.D. (ed.), *Amphibian Conservation*. Smithsonian, Washington, pp. 1-7.

Shinn, C.; Marco, A. & Serrano, L. 2008. Inter- and intra-specific variation on sensitivity of larval amphibians to nitrite. Chemosphere, 71: 507-514.

Shinn, C.; Marco, A. & Serrano, L. 2013. Influence of low levels of water salinity on toxicity of nitrite to anuran larvae. Chemosphere. http://dx.doi.org/10.1016/j.chemosphere.2013.01.079

Smith, G.R. & Dibble, C.J. 2012. Effects of an invasive fish (*Gambusia affinis*) and anthropogenic nutrient enrichment on American toad (*Anaxyrus americanus*) tadpoles. Journal of Herpetology, 46: 198-202.

Smith, G.R.; Temple, K.G.; Vaala, D.A. & Dingfelder, H.A. 2005. Effects of nitrate on the tadpoles of two ranids (*Rana catesbeiana* and *R. clamitans*). Archives of Environmental Contamination and Toxicology, 49: 559-562.

Storfer, A. 2003. Amphibian declines: future directions. Diversity and Distributions, 9: 151-153.

Tejedo, M. 2003. El declive de los anfibios. La dificultad de separar las variaciones naturales del cambio global, in: Rubio, X (ed.), *La conservación de los Anfibios en Europa*. Munibe 16:19-41.

Tilman, D.; Fargione, J.; Wolff, B.; D´Antonio, C.; Dobson, A.; Howarth R.; Schindler, D.; Schlesinger, W.H.; Simberloff, D.; Swackhamer, D. 2001. Forecasting agriculturally driven global environmental change. Science, 292: 281-284.

Trainer, D.O. & Karstad, L. 1960. Salt poisoning in Wisconsin wildlife. Journal of the American Veterinarian Medical Association, 13: 614-617.

Van Buskirk, J. & McCollum, A. 2000. Influence of tail shape on tadpole swimming performance. The Journal of Experimental Biology, 203: 2149-2158.

van Noordwijk, A.J. & de Jong, G. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. The American Naturalist, 128: 137-142.

Vidal-Abarca, M.R.; Montes, R.; Ramírez-Díaz, L. & Suárez, M.L. 1987. El clima de la cuenca del Río Segura (S.E. de España): factores que lo controlan. Anales de Biología, 12: 1-28.

Watkins, T.B. 1996. Predator-mediated selection on burst swimming performance in tadpoles of the Pacific tree frog, *Pseudacris regilla.* Physiological Zoology, 69: 154-167.

Westman, A.D.J.; Elliott, J.; Cheng, K.; van Aggelen, G. & Bishop, C. 2010. Effects of environmentally relevant concentrations of endosulfan, azynphosmethyl, and diazinon on Great Basin spadefoot (*Spea intermontana*) and Pacific treefrog (*Pseudacris regilla*). Environmental Toxicology and Chemistry, 29: 1604-1612.

Widder, P. D. & Bidwell, J.R. 2006. Cholinesterase activity and behavior in chlorpyrifos-exposed *Rana sphenocephala* tadpoles. Environmental Toxicology and Chemistry, 25: 2446-2454.

Widder, P. D. & Bidwell, J.R. 2008. Tadpole size, cholinesterase activity, and swim speed in four frog species after exposure to sub-lethal concentrations of chlorpyrifos. Aquatic Toxicology, 88: 9-18.

Wright, P.M. & Wright, P.A. 1996. Nitrogen metabolism and excretion in bullfrog (*Rana catesbeiana*) tadpoles and adults exposed to elevated environmental ammonia levels. Physiological Zoology, 69: 1057-1078.

Xu, Q. & Oldham, R.S. 1997. Lethal and sublethal effects of nitrogen fertilizer ammonium nitrate on common toad (*Bufo bufo*) tadpoles. Archives of Environmental Contamination and Toxicology, 32: 298-303.

FIGURE CAPTIONS

Fig. 1. Final mass and growth (g) (mean ± 1 SE) of larvae of *Pelophylax perezi* exposed to different nitrogenous treatments in the common garden experiment. Results of HDS Tukey´s test (for growth) and pairwise comparisons of estimated marginal means (for final mass) (α = 5% in all cases) for treatment are shown (common underbars notation). Treatment: 1) control; 2) 13.5 mg NH_4^+/L; 3) 13.5 mg NH_4^+/L + 364.7 mg NO_3^-/L + 6.67 mg NO_2^-/L; 4) 13.5 mg NH_4^+/L + 364.7 mg NO_3^-/L + 66.7 mg NO_2^-/L. Source populations: □ C1 (exposed to a reference environment); ◇ C2 (exposed to a reference environment); ▲ P1 (exposed to a polluted environment).

Fig. 2. Relationships between growth and larval speed for larvae exposed to different levels of nitrogenous pollution for 21 days in the common garden experiment. —□— Treatment 1 (control); ----◇---- Treatment 2 (13.5 mg NH_4^+/L); – –△– – Treatment 3 (13.5 mg NH_4^+/L + 364.7 mg NO_3^-/L + 6.67 mg NO_2^-/L); - - ◯ - - - Treatment 4 (13.5 mg NH_4^+/L + 364.7 mg NO_3^-/L + 66.7 mg NO_2^-/L).

Fig. 3. Mean (± 1 SE) mortality of larvae of *Pelophylax perezi* exposed to different field locations for 21 days in the field experiment. Locations: L1) reference; L2) polluted; L3) polluted. Source populations: □ C1 (exposed to a reference environment); ◇ C2 (exposed to a reference environment); ▲ P1 (exposed to a polluted environment).

Fig. 4. Final mass and growth (mean ± 1 SE) of *P. perezi* larvae raised at different field locations for 21 days in the field experiment. Results of HDS Tukey´s test (for growth) and pairwise comparisons of estimated marginal means (for final mass) (α = 5% in all

cases) for treatment are shown (common underbars notation). Locations: L1) reference; L2) polluted; L3) polluted. Source populations: □ C1 (exposed to a reference environment); ◇ C2 (exposed to a reference environment); ▲ P1 (exposed to a polluted environment).

Fig. 5. Relationships between growth and larval speed for larvae exposed to different field locations for 21 days in the field experiment. —□— L1 (reference);----◇---- L2 (polluted); – –△– – L3 (polluted destination field location).

Table 1. Summary statistics for AN(C)OVAs performed on larvae mass and growth after 21 days of exposure in the common garden and field experiments. All variables were log-transformed. ndf: numerator degrees of freedom; ddf: denominator degrees of freedom.

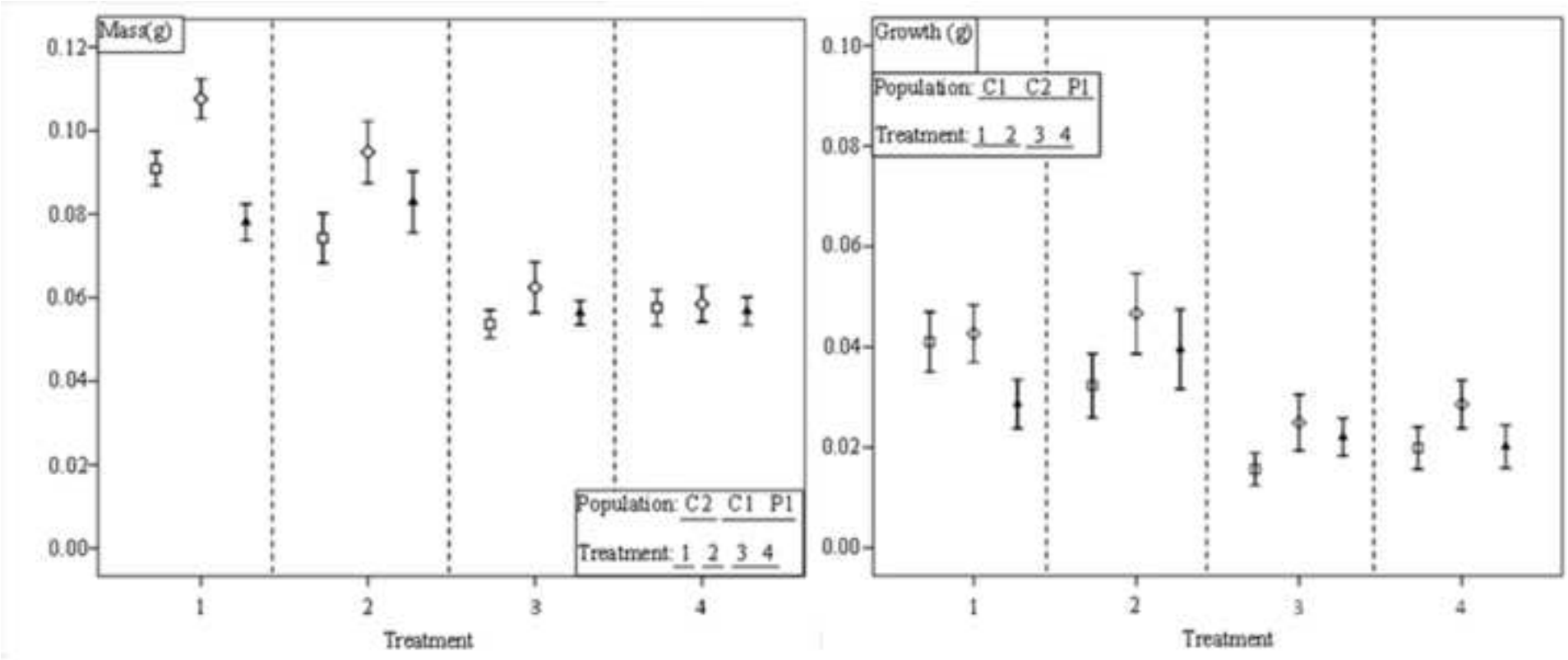
Source of variation	ndf	ddf	F	P	Source of variation	ndf	ddf	F	P
Common garden experiment					Field experiment				
					Final mass				
Initial mass	1	195	0.423	0.516	Initial mass	1	43	11.871	0.001
Population of origin	2	195	9.964	0.0001	Population of origin	2	43	1.425	0.252
Treatment	3	12.028	4.165	0.031	Location	2	10.701	17.900	0.0001
Pool(Treatment)	12	195	18.510	0.0001	Enclosure(Location)	6	43	0.336	0.914
Population x Treatment	6	195	2.487	0.024	Population x Location	4	43	0.123	0.974
					Growth				
Population of origin	2	196	3.115	0.047	Population of origin	2	44	1.863	0.167
Treatment	3	12.022	1.994	0.169	Location	2	5.395	34.330	0.001
Pool(Treatment)	12	196	5.350	0.0001	Enclosure(Location)	6	44	0.219	0.969
Population x Treatment	6	196	0.586	0.739	Population x Location	4	44	0.168	0.954

Table 2. Summary statistics for the regression analyses performed on swimming speed (dependent variable) and larval growth (independent variable) for the common garden and field experiments. All variables were log-transformed. See Material and Methods section for details on treatment codes. Note that, although they have the same value, p values for the "Regression analysis" and "B(S.E.)" columns correspond, respectively, to the significance of the model and to the coefficient for the independent variable.

Treatment	Regression analysis	B (S.E.)
	Common garden experiment	
1	Adjusted R^2= 0.076; $F_{1,56}$= 5.685; P= 0.021	-0.230 (0.097); P= 0.021
2	Adjusted R^2= 0.166; $F_{1,54}$= 11.938; P=0.001	0.132 (0.038); P=0.001
3	Adjusted R^2= 0.037; $F_{1,53}$= 3.072; P= 0.085	0.088 (0.050); P=0.085
4	Adjusted R^2= 0.043; $F_{1,49}$= 3.259; P= 0.077	0.108 (0.060); P=0.077
	Field experiment	
L1	Adjusted R^2= 0.098; $F_{1,21}$= 3.397; P= 0.079	-0.270 (0.146); P= 0.079
L2	Adjusted R^2= 0.203; $F_{1,17}$= 5.597; P= 0.030	0.313 (0.132); P= 0.030
L3*	Adjusted R^2= 0.000; $F_{0,16}$= -; P= -	-

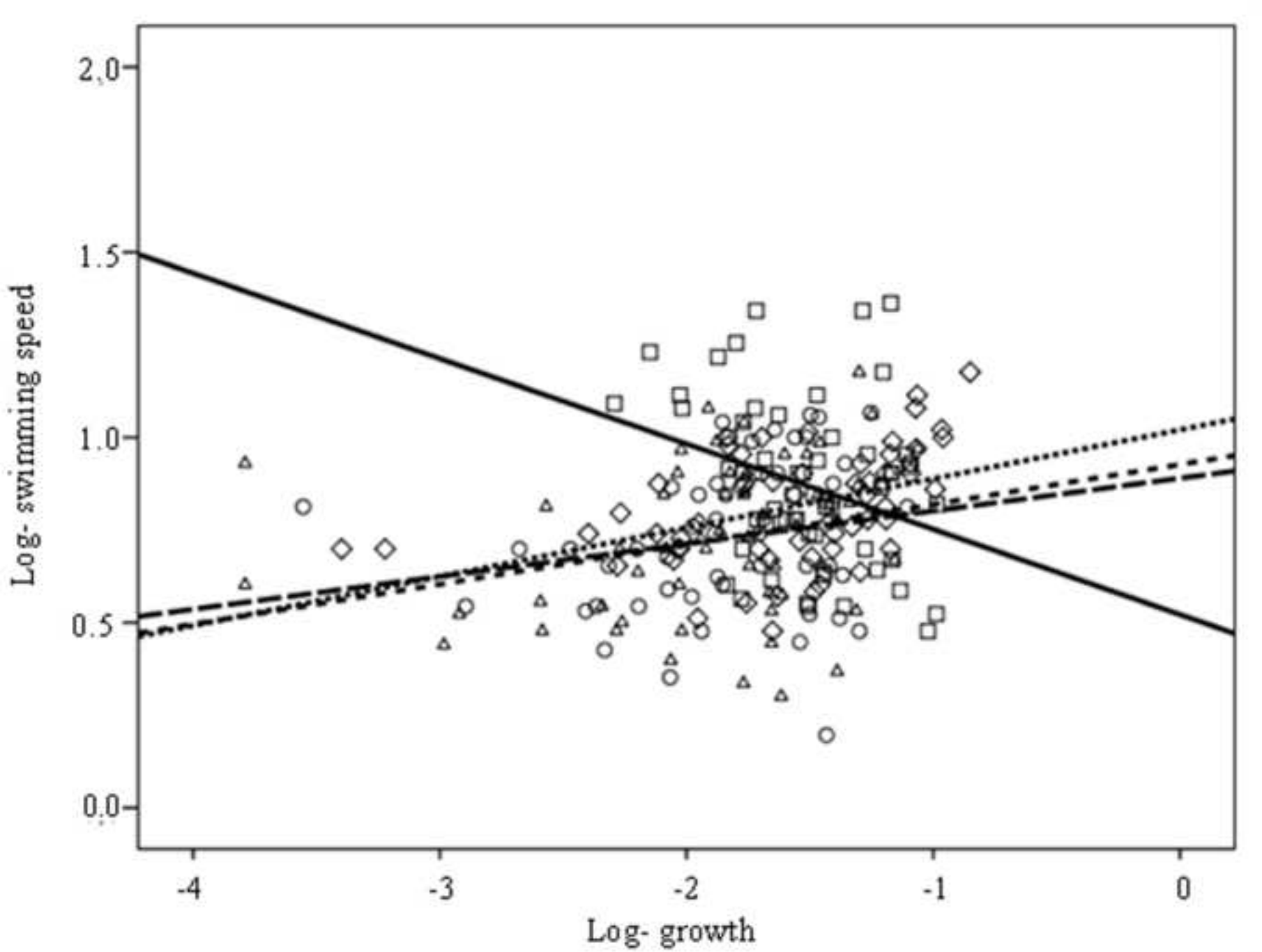
Figure(s)

Click here to download high resolution image



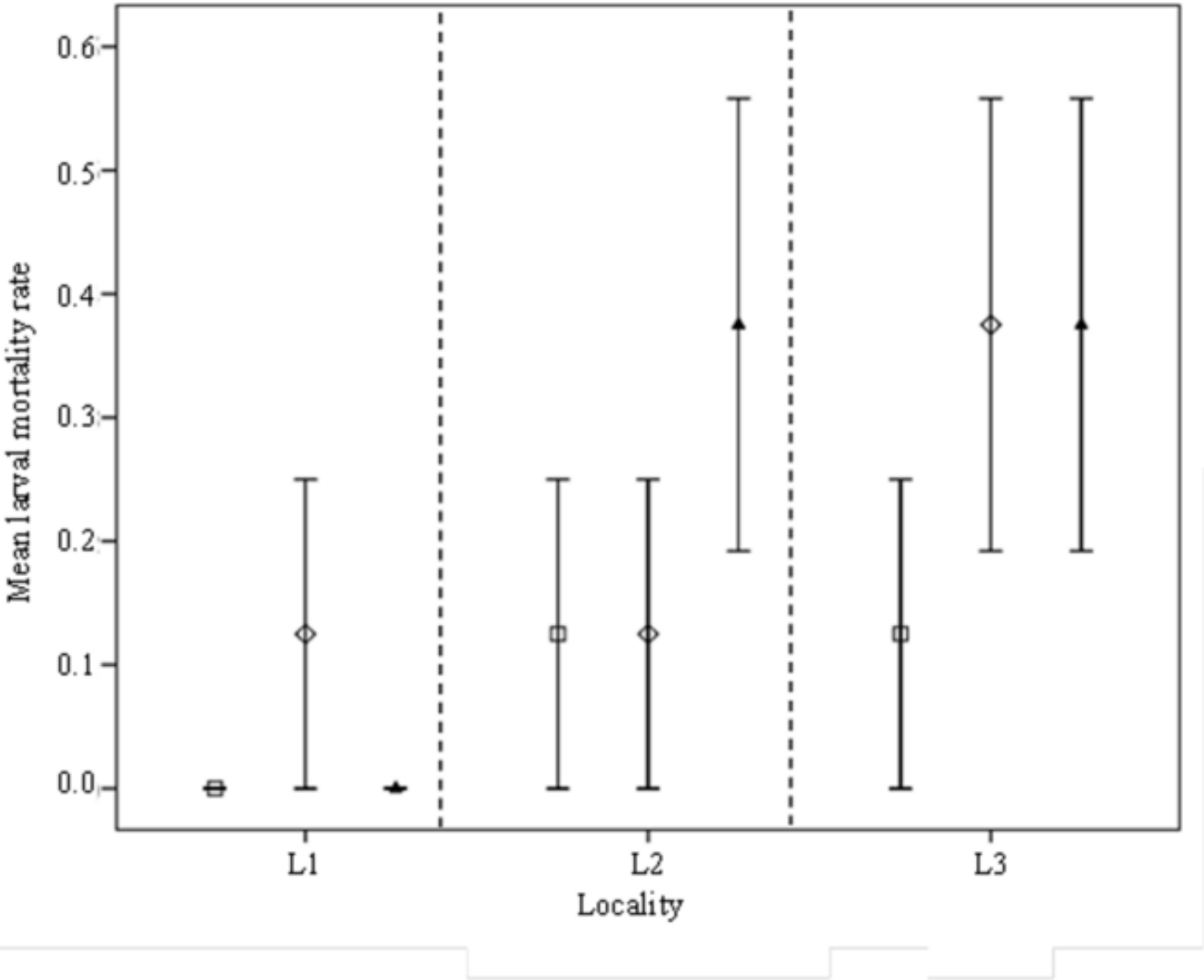
Figure(s)

Click here to download high resolution image



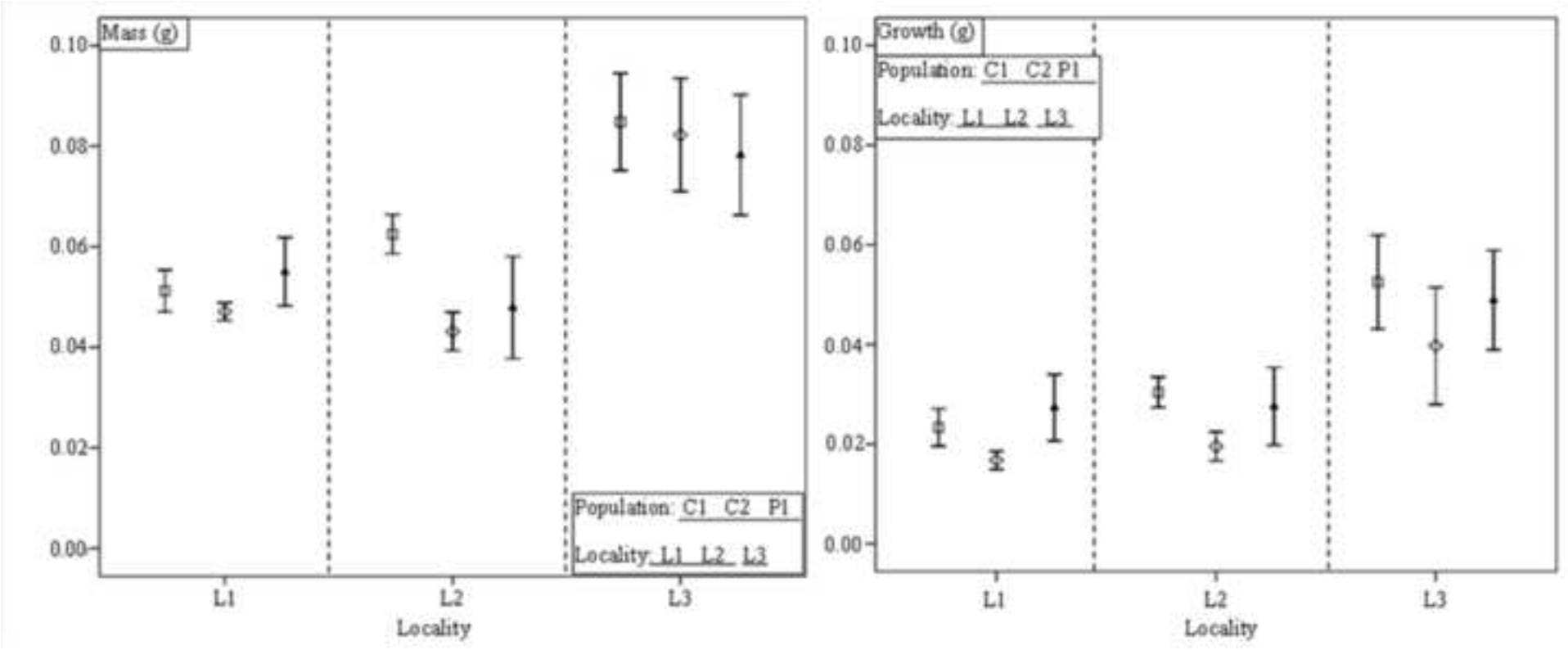
Figure(s)

Click here to download high resolution image



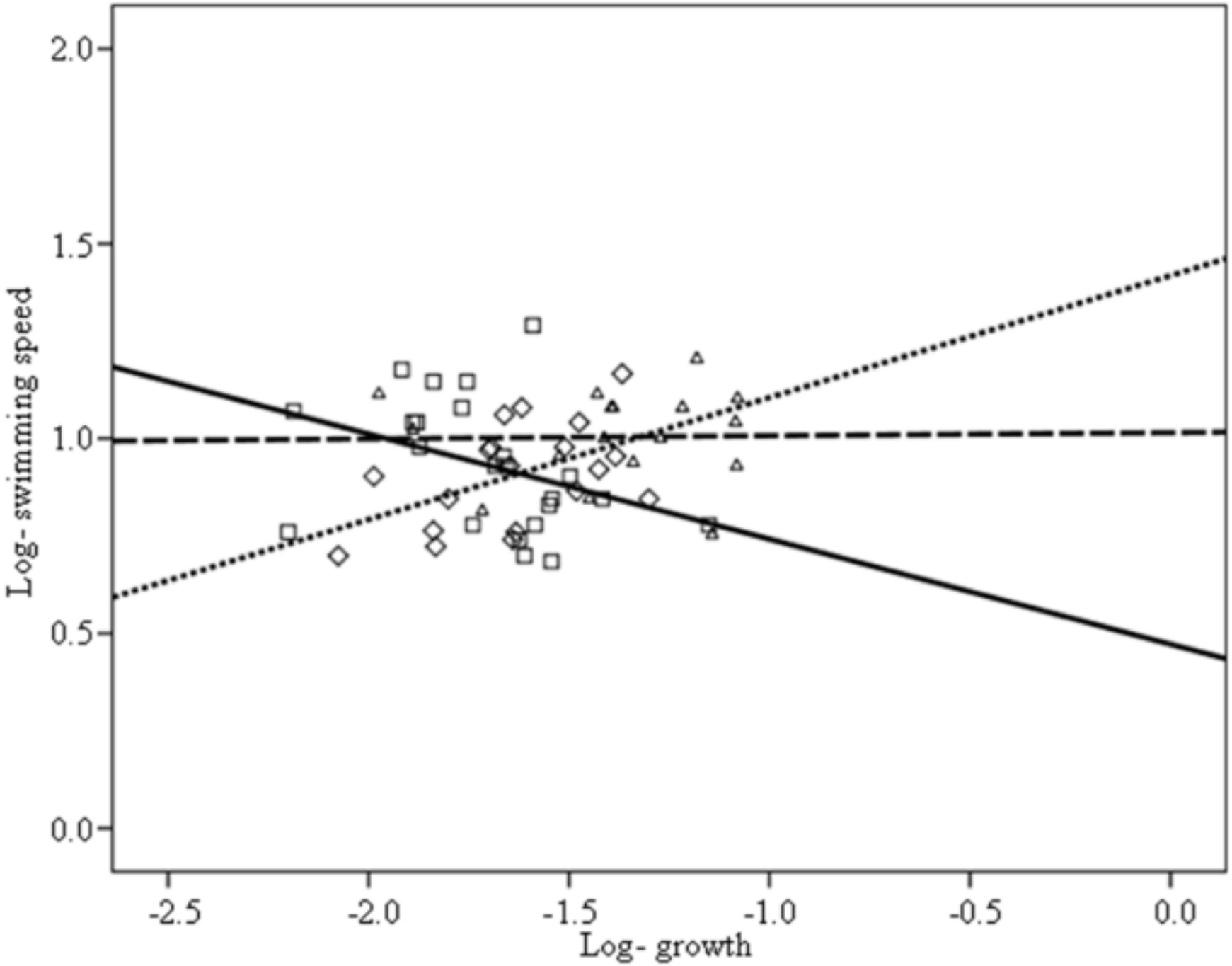
Figure(s)

Click here to download high resolution image



Figure(s)

Click here to download high resolution image



Supplementary Material

Click here to download Supplementary Material: Egea-Serrano&Tejedo-Aq_Toxicol_v5-DEF-Supplementary-Data-REVIEWED

HIGHLIGHTS

- We studied the effect of nitrogenous pollution on larval amphibians.
- We conducted experiments in semi-natural and natural venues.
- Pollution affects survival, fitness and the trade-off between growth and swimming.
- We found contrasting effects between experimental venues.
- The results suggest the convenience of accounting different environmental contexts.